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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.	ET 390094695 US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Jess G.	Thoene	New Orleans, LA

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Treatments for Cancer

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 17

☑ Drawing(s) *Number of Sheets* 8

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number _____

☐ Other (specify) _____

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

SIGNATURE _____

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Date 10/16/2003

REGISTRATION NO. *(if appropriate)* _____

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PTO/SB/17 (10-01)
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TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	USPS Express ET 390094695 US
Filing Date	10/16/2003
First Named Inventor	Jess G. Thoene
Examiner Name	
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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	80.00

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2. EXTRA CLAIM FEES

		Extra Claims	Fee from below	Fee Paid
Total Claims	-20** =		X	=
Independent Claims	- 3** =		X	=
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

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105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
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126	180	126	180	Submission of Information Disclosure Stmt	
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146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	J. Cale Lennon, III, Ph.D.	Registration No. (Attorney/Agent)		Telephone	(504) 585-6962
Signature				Date	10/16/2003

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PROVISONAL PATENT APPLICATION

TITLE OF THE INVENTION

"TREATMENTS FOR CANCER"

INVENTOR

Jess G. Thoene, a United States citizen, of New Orleans, Louisiana

CROSS-REFERENCE TO RELATED APPLICATIONS

Not Applicable

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

Not Applicable

FIELD OF THE INVENTION

The present invention relates to compounds useful for the treatment of cancer.

BACKGROUND

Cancer is an abnormal growth of cells. In particular, this abnormal growth is unresponsive to normal physiological restraints on cell growth. In some cases the production or growth of cells is warranted and necessary. In some cases, new cell growth is mandated by the death of existing cells. The death of cells can be random, caused by a myriad of conditions such as lack of nutrients, cellular damage, infection or exposure to toxins. However, in some cases the death of cells follows a controlled program. This phenomenon is known as apoptosis. Apoptosis is also known as planned cell death or controlled cell death. The death of cells by apoptosis differs in many respects from the death of cells by other means. Cells that die as a result of apoptosis exhibit hallmark molecular and physiological features that are distinct from those of cells hat die as a result of necrosis (Kanduc *et al.*, 2002). The process of apoptosis is characterized by several molecular features. One is the production of enzymes that causes cleavage of intracellular DNA that can be seen as a laddering pattern following gel electrophoresis (Shiokawa *et al.*, 2002). Apoptosis is also characterized by zeiosis or blebbing of cell nuclei (Coleman *et al.*, 2001). These features contrast with those associated with cell death by necrosis, which show no systematic breakdown of DNA and is usually accompanied by inflammation (Sauter *et al.*, 2000). Apoptosis has proven to be an intriguing option for those seeking additional methods of treating cancer because it is considered to be a more gentle treatment and likely to result is less unwanted damage to otherwise healthy cells.

Current treatments for cancer are centered on chemotherapeutic agents and radiation therapy. The goal of these two regimens is usually considered to be the killing of rapidly dividing cells by interfering with processes involved in the replication of cells. These treatments exploit the susceptibility of dividing cells to certain agents. Some of these methods include

treatments designed to damage microtubule assembly during mitosis such as colchicine and paclitaxel (Kaufmann and Earnshaw, 2000). There are other chemotherapeutic drugs that interfere with the replication of DNA such as doxorubicin and epirubicin (Buschini *et al.*, 2003). While these therapies are targeted to cancerous cells, they have similar effects on otherwise healthy cells involved in the cell division process. Therefore there is a need to develop alternate treatments for cancer that pose less of a danger to otherwise healthy cells.

Current examples of compounds that are used to treat cancer are usually considered to be effective due to their ability to interfere with the processes involved with cell division. However, there is growing evidence that some of these molecules are also able to induce apoptosis in the cancer and otherwise healthy cells (Kaufmann and Earnshaw, 2000). Researchers have been able to observe some of the characteristic features of apoptosis in cells subjected to currently used chemotherapeutic drugs. Many of the compounds used to treat cancer are also toxic to otherwise healthy cells. This is also true of some of the compounds used to induce apoptosis. The development of methods of treatment that utilize apoptosis as a treatment for cancer present a threat to otherwise healthy cells. Therefore methods of reducing the amount of an agent needed to induce apoptosis would be of great therapeutic benefit in the development of apoptotic cancer therapeutics.

Some of the molecules currently used to induce apoptosis are tumor necrosis factor alpha, ultraviolet light and Fas antibodies. These agents are routinely used to induce apoptosis in laboratory models of cancer. Several of the agents have limited suitability as possible treatment agents in individuals because of the physical nature of the treatment. For example ultraviolet light has only a limited ability to penetrate cells or the tissues of an individual. Some agents such as tumor necrosis factor alpha are peptides that require expensive synthesis and would

prove costly as treatments for individuals. Because of these limitations, any treatments that are able to enhance the apoptotic effect of known inducers of apoptosis or chemotherapeutic agents would be beneficial.

SUMMARY OF THE INVENTION

The present invention relates to novel compositions and methods for the treatment of various types of cancers. In one aspect of the invention, the invention involves compounds having the formula $[CH_3OR_1\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2.S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue.

In another aspect of the invention, the invention involves compounds having the formula $[CH_3OR_1\text{-}R_2\text{-}R_3 ....R_n\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and $R_2\,R_3 \ldots\ R_n$ are amino acid residues.

In another aspect of the invention the invention involves a method of treating cancer by administering to a patient a compound having the formula $[CH_3OR_1\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2.S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue.

In a further aspect of the invention, the invention involves a method of treating cancer in by administering to a patient a compound having the formula $[CH_3OR_1\text{-}R_2\text{-}R_3 ....R_n\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and $R_2\,R_3 \ldots\ R_n$ are amino acid residues.

In another aspect of the invention, the invention involves a method of treating cancer by administering to patient a compound having the formula $[CH_3OR_1\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2.S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and

where said compound is administered in conjunction with or prior to the administration of a chemotherapeutic agent.

In another aspect of the invention, the invention involves a method of treating cancer by administering to patient a compound having the formula $[CH_3OR_1\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and where said compound is administered in conjunction with or prior to the administration of a chemotherapeutic agent, said chemotherapeutic agent being an inducer of apoptosis.

In a further aspect of the invention, the invention involves a method of treating cancer by administering to a patient a compound having the formula $[CH_3OR_1\text{-}R_2\text{-}R_3 \ldots . R_n\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and $R_2 R_3 \ldots R_n$ are amino acid residues and where said compound is administered in conjunction with or prior to the administration of chemotherapeutic agent.

In a further aspect of the invention, the invention involves a method of treating cancer by administering to a patient a compound having the formula $[CH_3OR_1\text{-}R_2\text{-}R_3 \ldots . R_n\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and $R_2 R_3 \ldots R_n$ are amino acid residues and where said compound is administered in conjunction with or prior to the administration of chemotherapeutic agent, said chemotherapeutic agent being an inducer of apoptosis.

In another aspect of the invention, the invention involves a method of treating cancer by administering to a patient the compound cystine dimethyl ester.

In another aspect of the invention, the invention involves a method of treating cancer by administering to a patient the compound cystine dimethyl ester in conjunction with or prior to the administration of a chemotherapeutic agent.

In another aspect of the invention, the invention involves a method of treating cancer by administering to a patient the compound cystine dimethyl ester in conjunction with or prior to the administration of a chemotherapeutic agent, said chemotherapeutic agent being an inducer of apoptosis.

DETAILED DESCRITPITON OF THE INVENTION

The present invention involves compounds and methods for treating cancer. In accordance with the present invention, cysteine dimethyl ester (CDME) is administered to a patient diagnosed with cancer as part of a therapeutic regimen that includes one or more chemotherapeutic agents. As a preferred embodiment, the chemotherapeutic agent is a compound that induces apoptosis. In another preferred embodiment, the cancer is an epithelial-derived cancer. Examples of epithelial derived cancers include renal cell carcinoma and pancreatic cancer.

Another aspect of the present invention involves novel compounds having the formula $[CH_3OR_1\text{-}R_2\text{-}R_3\ldots.R_n\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and $R_2\,R_3\ldots\,R_n$ are amino acid residues. As a preferred embodiment, the compound has the formula $[CH_3OR_1\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from either an aspartate or glutamate amino acid residue. As an example, the compound aspartyl-cystamine disulfide is shown in Figure 7.

Another aspect of the present invention involves administering a compound having the formula $[CH_3OR_1\text{-}R_2\text{-}R_3\ldots.R_n\text{-}NH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}S]_2$ where CH_3OR_1 is a terminal ester derived from an aspartate or glutamate amino acid residue and $R_2\,R_3\ldots\,R_n$ are amino acid residues, to a patient diagnosed with cancer as part of a therapeutic regimen that includes one or

more chemotherapeutic agents. As a preferred embodiment, the compound has the [CH_3OR_1 - NH_2-CO-NH-CH_2-CH_2 .S]$_2$ where CH_3OR_1 is a terminal ester derived from either an aspartate or glutamate amino acid residue. As further a preferred embodiment, the chemotherapeutic agent is a compound that induces apoptosis. In another preferred embodiment, the cancer is an epithelial-derived cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The effect of MEA or cystine dimethylester (CDME) on apoptosis in semi-confluent nephropathic cystinotic or normal fibroblasts. Apoptosis was assayed by CaspACE. Nephropathic cystinotic fibroblasts (passage 7–13) were treated with cysteamine 1 hour before exposure to apoptotic triggers, depleting them to a normal lysosomal cystine content (0.01 to 0.13 nmol of cystine per mg of protein). Normal fibroblasts also at passage 7–13 were pretreated with 0.5mM CDME for 1 hour before apoptotic triggers, loading their lysosomes with cystine to the cystinotic range (0.47 to 1.95 nmol of cystine per mg of protein). They were then treated with apoptotic stimuli and incubated for 16 hours while in cystine-free or CDME-containing medium. Apoptosis was assayed by CaspACE.

Figure 2. (A through D) The effect of lysosomal cystine in normal and nephropathic cystinotic fibroblasts. Nephropathic cystinotic fibroblasts were exposed to UV light (60 mJ) (A) or pretreated with 1.0 mM MEA for 1 h and then exposed to UV light (60 mJ) (B). Normal fibroblasts were exposed to UV light (60 mJ) (C) or pretreated with 0.5 mM CDME for 1 hour and then exposed to UV light (60 mJ) (D). Apoptosis was then assessed by CaspACE and photographed by fluorescent microscopy. The cystine content of parallel plates was as follows:

A, 4.0 nmol/mg protein; B, 0.01 nmol/mg protein; C, <0.01 nmol/mg protein; D, 1.95 nmol/mg protein.

Figure 3. The effect of CDME or MEA on the morphology of normal or nephropathic cystinotic fibroblasts. Fibroblasts were cultured under the usual conditions and exposed to TNF-α for 16 hours as described in Materials and Methods. Panels a through c are normal fibroblasts; panels d through f are nephropathic cystinotic fibroblasts. Panels b and e show cells after TNF-α exposure alone; panels c and f show the effect of CDME in normal cells (c) or MEA in cystinotic cells (f). Note typical apoptotic morphology in panels b, c, and e, with more apoptotic cells in e (cystinotic) than b (normal). Enhanced apoptosis is seen in normal cells to which CDME is added (c). More normal morphology is seen in nephropathic cystinotic cells after MEA treatment (f). Photographs via phase microscopy.

Figure 4. Loss of colocalization of cathepsin B in normal and nephropathic cystinotic fibroblasts after TNF-α exposure. Fibroblasts were maintained under normal culture conditions, exposed to TNF-α, loaded with Lysotracker red, fixed, and stained for cathespin B using an anti-cathepsin B antibody as described in Materials and Methods. Panels a through f are normal fibroblasts; a through c are control cells; d through f are treated with TNF-α. Panels g through l are cystinotic fibroblasts; g through i are control; j through l are treated with TNF-α.

Figure 5. The effect of CDME on apoptosis in renal proximal tubule epithelial (RPTE) cells. Semi-confluent RPTE cells were treated with either 0.25 mM CDME or TNF-α, harvested at 0.5, 1, 3, and 6 hours, stained with TUNEL, and enumerated by fluorescence microscopy.

Figure 6. The effect of CDME on apoptosis in RPTE cells. Fluorescent micrographs of the cells in Figure 5 are shown at 0.5, 1, 3, and 6 hours (A through D, respectively).

Figure 7. Aspartyl-cystamine disulfide.

Figure 8. Effect of Various Compounds on Rate of Apoptosis.

EXAMPLES

Cystine Binding Protein Assay

Intracellular lysosomal free cystine was determined using a cystine binding protein (CBP) assay as described. CBP was procured from Riverside Scientific. The assay has a sensitivity of 0.1 μM. Total cell protein was determined by a modification of the Lowry method . Statistical analyses were performed using paired *t* test for means with SSPS for Windows.

Immunhistochemistry of Normal and Cystinotic Fibroblasts

Cells were stained with Lysotracker Red (Molecular Probes) for 5 min at room temperature, washed twice in PBS, and then fixed in 4% formalin for 1 hour. Slides were immersed in blocking buffer (PBS, pH 7.2, 0.5% Carnation dry milk, 0.1% Triton X-100) for 20 min at room temperature, washed twice in PBS, and then incubated with primary antibody (anti-cathepsin B, Santa Cruz Biotechnologies) diluted 1:100 for 2 hours at room temperature, followed by incubation with secondary antibody (FITC-conjugated rabbit anti-goat, Santa Cruz Biotechnologies), at 4°C overnight. Slides were washed twice with PBS and then sealed with cover slips and viewed using a Leica DMRX deconvoluting microscope.

Effect of Various Compounds on Rate of Apoptosis

Cells were incubated with various apoptotic compounds at a concentration of 0.5 mM for 16 hours. The structures of the treatment compounds are shown in Figure 8. Following incubation, cells were stained with CaspASE. Fluorescence-activated cell sorting (Beckman Coulter Epics Elite) was used to determine the percentage of cells undergoing apoptosis from each treatment group.

Cell Culture

Normal and cystinotic fibroblasts were purchased from The Coriell Mutant Cell Repository, and cultured in Coon modification of Ham F12 medium, supplemented with 10% fetal calf serum (FCS). Renal proximal tubule epithelial (RPTE) cells were purchased from Biowhittaker, cultured in renal epithelial basal medium supplemented with one Singlequots kit per 500 ml to make renal epithelial growth media (REGM, Biowhittaker). Fibroblasts and RPTE were maintained in a 5% carbon dioxide, 95% air, humidified incubator at 37°C. Induction of apoptosis and assays for its detection were performed using commercially available reagents. Normal and cystinotic fibroblasts were matched for passage number and cell density, and then exposed to one of three apoptotic triggers: TNF-α (2 ng/ml) with actinomycin D (2.5 μg/ml) for 16 h; anti-Fas antibody (500 ng/ml) with actinomycin D (2.5 μg /ml) for 16 h; or UVB light (60 mJ). After exposure, the cells were maintained in Coon modification of Ham F12 medium for 16 hours before analysis. The cells were then assayed for apoptosis. Serum withdrawal was also used as an apoptotic stimulus, in which case the cells were incubated in F12 medium without serum for 24 hours and then analyzed for apoptosis as described. Three commercially available

apoptosis assays were employed. CaspACE (Promega) is an FITC-conjugated cell-permeable form of the pan-caspase inhibitor zVAD-Fmk, which binds to activated caspase(s).

Cells were incubated in FITC-VAD-Fmk–containing medium (10 μM for 30 minutes at 37°C), washed, and then fixed in 10% buffered formalin (30 minutes at room temperature) before analysis. TUNEL employs terminal deoxynucleotidyl transferase (TdT) to label the ends of double-stranded DNA breaks, which occur in apoptotic cells, with FITC-conjugated dUTP. Cells were fixed in 4% buffered formalin, washed, incubated in permeabilization solution (0.1% Triton X-100 in 0.1% Na Citrate) for 2 minutes on ice and then stained with 25 μl of TdT/45 μl of labeled dNTP mix for 40 min at 37°C. Annexin V-propidium iodide (PI) staining was performed as described (Annexin V Fluos kit directions, Roche Laboratories). The cells were visually enumerated by fluorescence microscopy, scoring a minimum of five fields (250 to 300 cells) for fluorescence in triplicate followed by counting all cells in the same field by light microscopy, with a minimum of 750 cells scored per condition. The apoptosis rate is the total number of cells that fluoresce divided by the total cells in the field.

Lysosomal cystine depletion of cystinotic fibroblasts was accomplished by treatment with 1 mM cysteamine-HCl(Sigma) in Ham F12 medium lacking cystine (Life Technologies), with 10% FCS, for 1 hour followed by exposure to the apoptotic triggers in cystine-free medium to inhibit cystine re-accumulation. Normal fibroblast lysosomes were loaded with cystine by the addition of 0.5 mM cystine dimethylester (CDME, Sigma) to normal culture medium for 1 hour before treatment. The cells were treated with apoptotic triggers as described above, leaving CDME in the medium to prevent lysosomal cystine loss, and then analyzed for apoptosis as described. Lysosomal cystine loading of RPTE cells was accomplished by exposure to 0.1 mM or 0.25 CDME in normal REGM for 1 hour before treatment with or without apoptotic triggers.

Results

The cell lines studied, their genotypes, and nominal cystine content are listed in Table 1. The mutations in cell lines GM00008, GM00760, and GM00046 cause typically severe nephropathic cystinosis with ESRD by 10 yr of age. The cystine content shown in the cystinotic lines in Table 1 varies between 0.8 and 15.7 nmol/mg protein, which is that typically seen in cultured cystinotic fibroblasts. Apoptosis induced in three nephropathic cystinotic, two normal, one intermediate cystinotic, and one ocular cystinotic fibroblast line by three separate inducers is shown in Tables 2 and 3. In Table 2, apoptosis in these lines was assessed by CaspACE. The cystinotic cells on average display about 2 to 3 times the apoptotic rate for the three apoptotic triggers compared with the normal cell lines. The cystinosis variant lines do not show increased apoptosis under these conditions. The differences are statistically significant at P +/- 0.05 between the averages for cystinotic and normal lines and between nephropathic and ocular cystinotic lines. There is no significant difference in the rate of apoptosis between intermediate and nephropathic lines, ocular cells and normal cells, or intermediate *versus* normal cells (Table 2). Similar results were obtained when the cells were analyzed by TUNEL, except that line GM00008 did not differ significantly from the normal lines' response after anti-Fas or UV exposure, nor did line GM00046 after TNF-α exposure (Table 3). Again, the variant lines did not show augmented apoptosis compared with the normal fibroblast lines.

Table 1. Genotype and cystine content of cell lines

Cell Line	Phenotype	Genotype	Cystine Content (nmol/mg protein)
GM00008	Nephropathic	46XX, 65-kb del	7.5
GM00760	Nephropathic	46XY, 753 G3A, premature stop	0.78
GM00046	Nephropathic	46XY, 5-bp del, frameshift	1.51
GM08761	Ocular	46XX, not determined	6.29
GM00379	Intermediate	IVS11_2 T3C	15.7
GM00010	Normal	46XY, apparently normal	0.01
GM05399	Normal	46XY, apparently normal	0.01
RPTE	Normal	46XY, apparently normal	0.6

Table 2. The apoptosis rate in cystinotic nephropathic, variant, and normal fibroblasts

		% Apoptosis			
		TNF-α_	Anti-Fas	UV	Control
GM008	Nephropathic	14.9	17.7	12.8	2.2
GM760	Nephropathic	13.5	14.4	26.3	3.1
GM046	Nephropathic	16.1	22.3	13.1	2.2
Average		14.8	18.1	17.4	2.5
GM08761	Ocular	8.2	5.9	6.4	2.3
GM00379	Intermediate	11.4	7.7	8.4	3.1
GM010	Normal	9.2	6.5	7.0	2.8
GM05399	Normal	6.3	4.9	7.2	1.9
Average		7.8	5.2	7.1	2.4

Table 3. The apoptosis rate in normal and cystinotic fibroblasts cell

		%Apoptosis			
	Phenotype	TNF-α	Anti-Fas	UV	Control
GM00008	Nephropathic	17.1	12.3	13.7	3.1
GM00760	Nephropathic	19.3	17.7	21.6	2.4
GM00046	Nephropathic	15.3	15.9	19.2	2.6
Average	Nephropathic	17.2	15.3	18.2	2.7
GM08761	Ocular	5.5	7.1	6.8	3.4
GM00379	Intermediate	8.2	8.5	8.5	2.8
GM00010	Normal	12.8	9.6	11.9	2.4
GM05399	Normal	11.4	10.5	7.6	2.4
Average	Normal	12.1	10.1	9.8	2.4

Modulation of the apoptotic response by altering the lysosomal cystine content of normal or nephropathic cystinotic fibroblasts is shown in Figure 1. The bar graphs show the apoptosis rates induced by exposure of two cystinotic and two normal cell line cells to TNF-α or UV radiation before and after correction of the cystine content of nephropathic fibroblasts to normal levels with MEA and before and after increasing the cystine content of normal fibroblasts to cystinotic levels by pre-incubation with CDME. The mean rate of apoptosis for the cystinotic cells before cystine depletion was 15.6 +/- 2.7%; after cystine depletion by exposure to MEA, it fell to 6. This difference is significant at $P < 0.001$. The average control apoptotic rate for the normal fibroblast lines was 7.2 +/- 1.3%, which rose to 18.7 +/- 5.4% after exposure to CDME ($P< .001$). The effect holds whether induction was by TNF-α or UV light. Representative fluorescence micrographs displaying this effect after UV exposure are shown in Figure 2. The

high rate of apoptosis in cystinotic fibroblasts with initial cystine content of 4.0 nmol/mg protein is shown in Figure 2A. This fell after treatment with cysteamine, which lowered the cystine content to < 0.1 nmol/mg protein (Figure 2B). The normal rate of apoptosis in normal fibroblasts (cystine content <0.1 nmol/mg protein) is shown in Figure 2C, and this increases to a rate similar to that seen in cystinotic fibroblasts after pre-exposure of the cells to CDME (Figure 2D), which increased the cystine content to 1.95 nmol/mg protein. Representative phase micrographs of fibroblasts treated with TNF-α, showing characteristic apoptotic morphology, and the effect of lysosomal cystine on apoptosis are shown in Figure 3, in which typical blebs in the plasma membrane are seen after treatment with TNF-α in both normal and cystinotic cell lines and the modulating effects of MEA and CDME on apoptosis are seen. Serum withdrawal in cultured fibroblasts caused less apoptosis above baseline than the other stimuli employed; however, increasing lysosomal cystine, either naturally due to defective CTNS function or artificially due to CDME loading, again resulted in an increased apoptotic rate (Table 4).

Table 4. Apoptosis in normal and nephropathic cystinotic fibroblasts exposed to serum withdrawal

Condition	%Apoptosis	
	GM00008	GM05399
Untreated controls	2.8	3.1
Serum withdrawal	8.7	6.1
Serum withdrawal and MEA	7.2	--
Serum withdrawal and CDME	--	37.3

To substantiate that lysosomes are permeabilized by TNF-α under these conditions, cathepsin B (a lysosomal cysteine protease) was localized by immunohistochemistry in normal and nephropathic cystinotic cells before and after induction of apoptosis. Lysosomes were identified with Lysotracker red dye. In both normal and cystinotic fibroblasts before TNF-α treatment, cathepsin B (green) displays a punctuate pattern that is closely associated with the red

lysosomal dye. Induction of apoptosis by TNF-α causes a translocation of cathepsin B from a lysosomal location to a diffuse cytosolic location, with loss of co-localization of color (Figure 4).

Cultured human renal proximal tubule epithelial cells display marked sensitivity to apoptosis after CDME exposure (Table 5). The concentration employed in fibroblasts (0.5 mM) was toxic to these cells and was decreased to 0.1 or 0.25 mM for the RPTE experiments. The rate of apoptosis produced by 0.25 mM CDME alone was equal to that induced by exposure to TNF-α alone (Figure 5). The time course was accelerated, with these cells attaining a maximum rate of apoptosis within 6 hours, (as opposed to 17 hours in fibroblasts) followed by lysis and release from the culture dish. The RPTE cystine content after exposure to 0.25 mM CDME for 1 hour was 1.99 nmol/mg protein. Representative fluorescence micrographs at each time point are shown in Figure 6.

Table 5. Percentage of apoptosis induced by selected compounds

Compound	% Apoptosis
Control	5.3
Cystine	8.3
CDME	39.7
Cystamine	5.0
Djenkolic Acid	5.8
DKADME	6.0
Penicillimine disulfide	4.5
PDDME	5.8
Cys-ala disulfide	5.1
CADME	4.8
Cys-gly disulfide	4.4
CGDME	7.4
Cys-val disulfide	6.3
CVDME	4.5

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CLAIMS

I claim:

1. A method of treating cancer consisting of administering to a patient cystine dimethyl ester, and a pharmaceutically acceptable carrier.

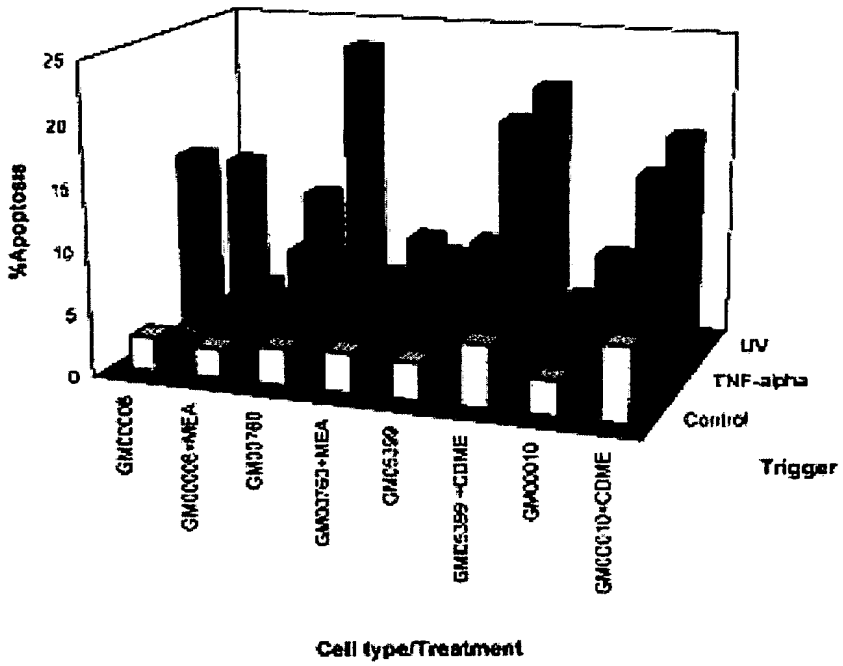

25
20
15
10
5
0
%Apoptosis
GM00008+MEA
GM03752+MEA
GM05399 +CDME
GM00010
GM00010+CDME
UV
TNF-alpha
Control
Trigger
Cell type/Treatment

FIGURE 1

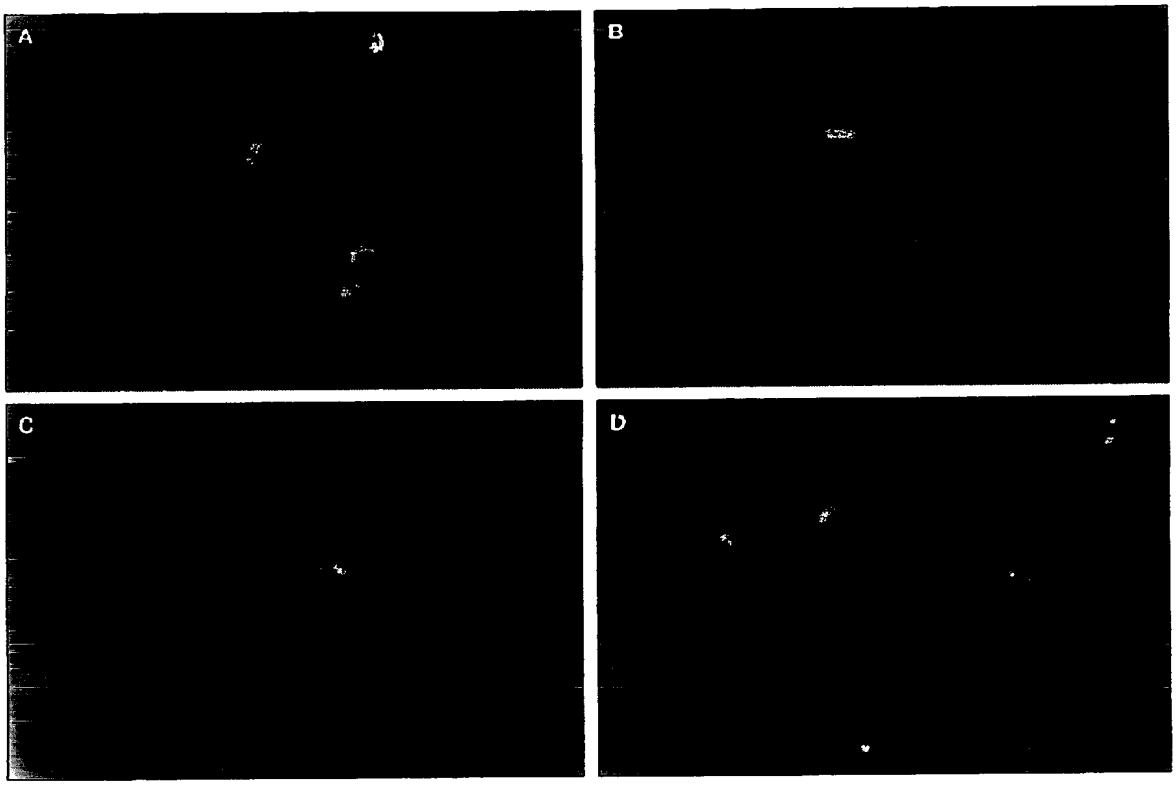


FIGURE 2

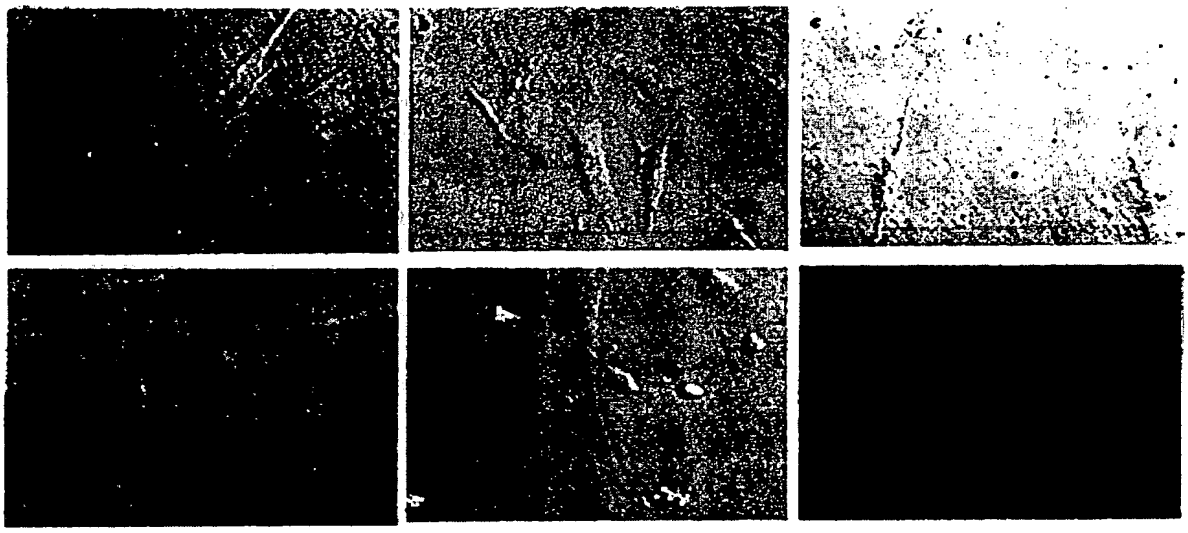


FIGURE 3

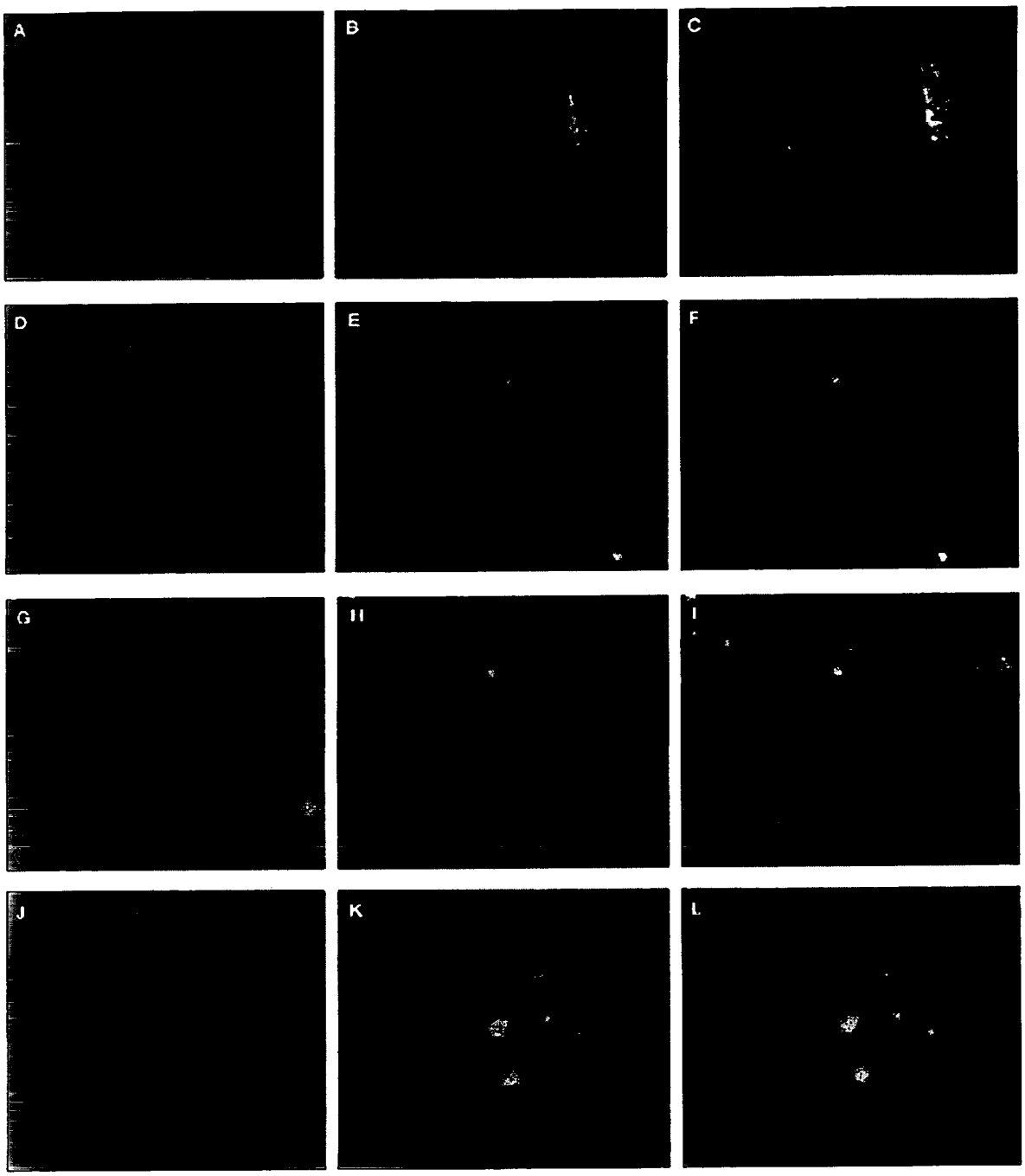


FIGURE 4

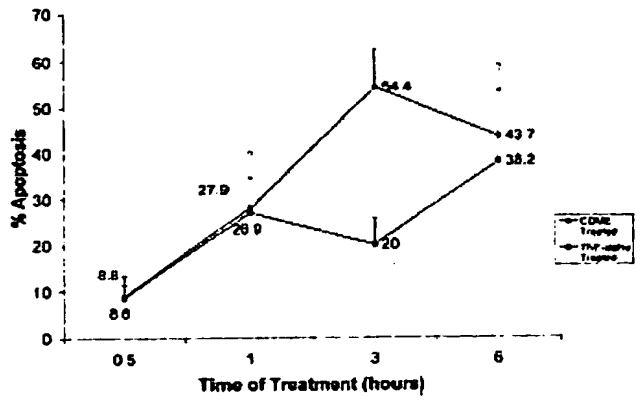

70
60
50
40
30
20
10
0
% Apoptosis
8.8
8.6
27.9
28.9
54.4
20
43.7
38.2
0.5
1
3
6
Time of Treatment (hours)

FIGURE 5

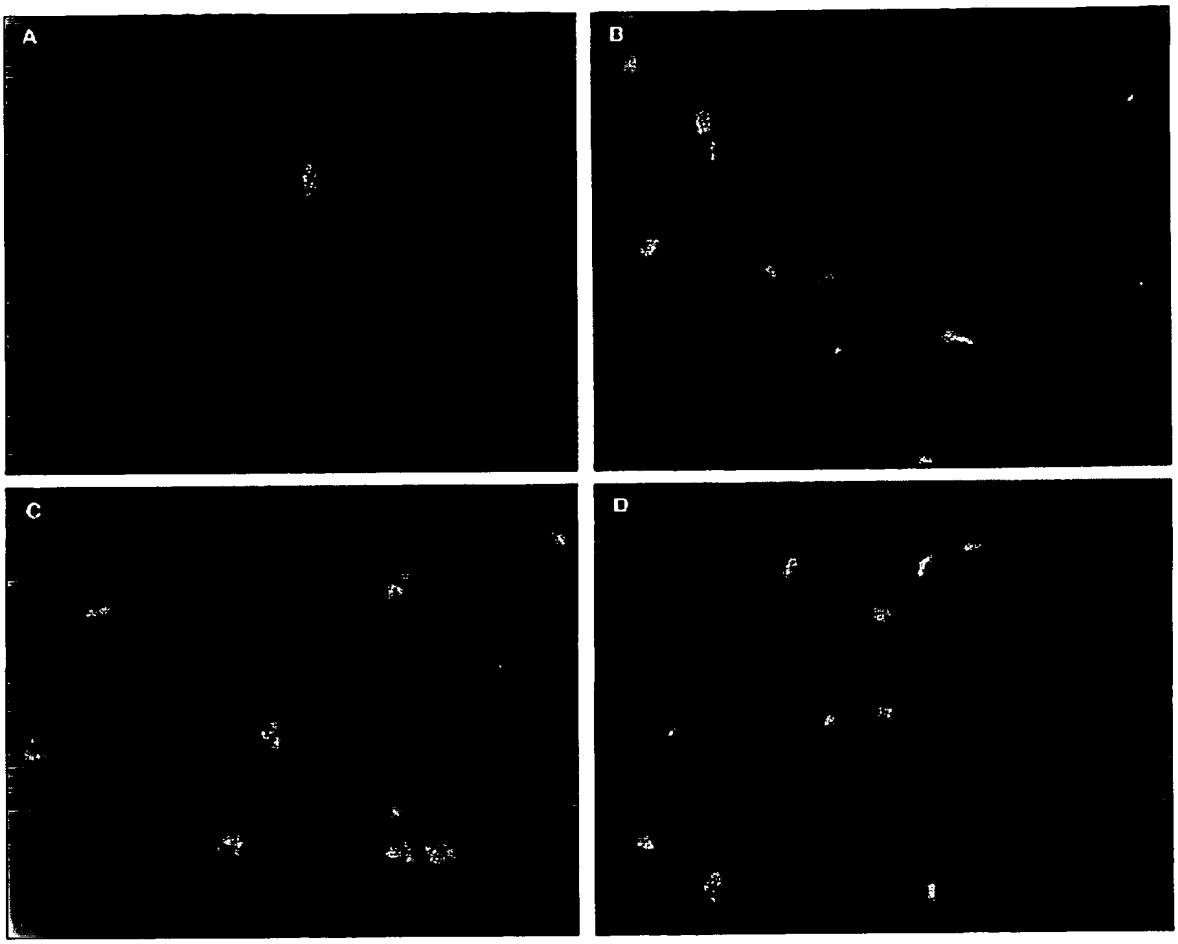


FIGURE 6

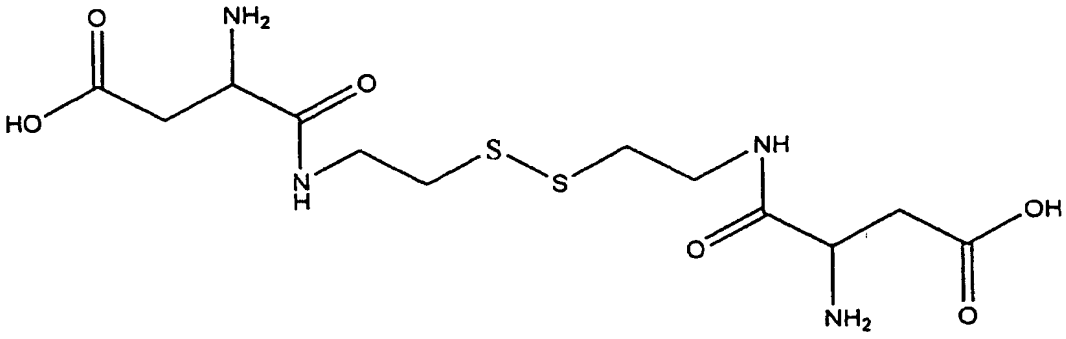


FIGURE 7

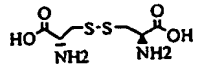
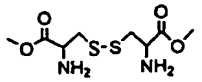
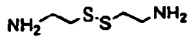
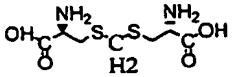
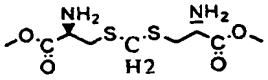
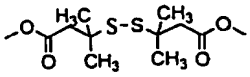
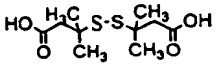
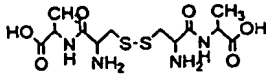
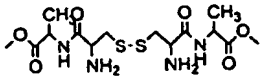
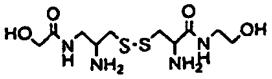
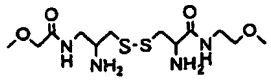
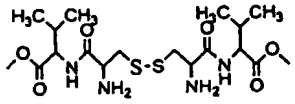
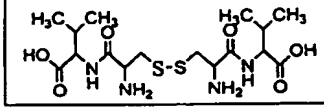
Structure	Treatment	% Apoptosis
	Control	5.3
	Cystine	8.3
	CDME	39.7
	Cystamine	5.0
	Djenkolic Acid	5.8
	DKADME	6.0
	Penicillimine disulfide	4.5
	PDDME	5.8
	Cys-ala disulfide	5.1
	CADME	4.8
	Cys-gly disulfide	4.4
	CGDME	7.4
	Cys-val disulfide	6.3
	CVDME	4.5

FIGURE 8

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